Anxiety during the postpartum period: examining the role of GABA in the medial prefrontal cortex

Undergraduate Honors Research Thesis

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Abstract

The postpartum period is commonly accompanied by emotional changes, which for many new mothers includes a reduction in anxiety. Previous research on the postpartum reduction in anxiety in rodents has shown that it is dependent on offspring contact and further has implicated enhanced GABAergic neurotransmission as an underlying mechanism. However, the specific brain regions where GABA acts to regulate the offspring-induced reduction in postpartum anxiety requires further investigation. Of particular interest is the medial prefrontal cortex (mPFC), which has been shown to play a role in the modulation of anxiety-related behavior. Here, we test the hypothesis that offspring interactions reduce anxiety-like behavior in postpartum females (dams) via GABA signaling in the mPFC in three experiments. In experiment one, we confirmed that dams display reduced anxiety compared to virgin females when tested on the elevated plus maze, an effect which was abolished by infusion of the $GABA_A$ receptor antagonist bicuculline in the mPFC. In experiment two, we found that dams that had been separated from their pups for 4 hr displayed increased anxiety as compared to dams that were not separated. Furthermore, activation of $GABA_A$ receptors in the mPFC by the agonist muscimol restored the reduced levels of anxiety-like behavior. In a final experiment, we found that mothers that were separated from their pups not only show increased levels of anxiety-like behavior but also had a lower number and percentage of activated GABAergic neurons within the mPFC. Together, these results suggest that mother-pup interactions reduce anxiety in postpartum females via $GABA_A$ neurotransmission in the mPFC and in doing so provide insight into mechanisms that may become dysfunctional in high postpartum anxiety.

Introduction

The postpartum period is marked by substantial differences in behavior of the mother, both towards the infant, and also towards the outside world (Murray and Cooper., 1997). These changes are thought to be a result of both psychological and physiological adjustments after birth. During the postpartum period mothers not only have to recover physically from pregnancy, but must develop behaviors that enable her to create the necessary bond with the child, which allows her to be aware of their needs in addition to her own (Walker et al., 1986). Most commonly, motherhood in both humans and rodents is accompanied by decreased anxiety behaviors, which is likely to be adaptive and increase her ability to cope with the stressors associated with raising a newborn (Fleming and Luebke., 1981; Hard and Hansen., 1985; Lonstein, 2005; Lonstein., 2007). In order for the reduction in maternal anxiety to take place, mothers must be able to interact with their young, specifically through physical contact (Lonstein, 2005; Lonstein, 2007). However, the period after birth is also a time when about 10-15% of mothers develop anxiety disorders (Fairbrother et al., 2016; Dennis et al., 2017; Pawluski et al., 2017). Elevated anxiety after birth may interfere with interaction between a mother and child, thus impeding the development of the offspring (Vallee et al., 1997; Brouwers et al., 2001; Weinstock, 2005).

GABA is the main inhibitory neurotransmitter in the brain and there is an abundance of research implicating GABAergic neurotransmission as a mediator of anxiety behavior in both non-maternal rodents and humans (Kalueff and Nutt, 2007; Smith and Rudolph, 2012; Gauthier, 2015). Likewise, GABA has been linked to the postpartum suppression of anxiety-related behavior that is mediated by physical contact with offspring (Lonstein, 2007; Lonstein et al., 2014). For example, in laboratory rats, cerebrospinal fluid concentrations of GABA were shown to be higher in lactating rats that interact with pups, but were low in those dams who has been separated from

their pups (Qureshi et al., 1987). However, other than the periaqueductal gray (PAG) (Miller et al., 2010), the specific brain regions where GABA acts to regulate postpartum anxiety is largely unknown and thus requires further investigation. Of particular interest in the medial prefrontal cortex (mPFC), which has been previously associated in modulation of anxiety-like behavior in rodents and humans (Jaferi and Bhatnagar, 2007; Lacroix et al., 2000; Stern et al., 2010; Adhikari, 2014; Calhoon and Tye, 2015). The mPFC, which is rich in GABAergic neurons, has been shown to have elevated basal GABA release and turnover in postpartum rats, higher expression of the GABA precursor GAD65, as well as greater expression of vesicular GABA transporter when compared to virgins, which together indicate a greater potential for cortical GABA synthesis and release in mothers (Kornblatt and Grattan, 2001; Ahmed et al., 2012; Arriaga- Avila et al., 2014). Thus, we hypothesize that enhanced GABAergic signaling occurs in the mPFC as a result of offspring contact and contributes to the reduction in anxiety-like behavior in postpartum females.

To test this hypothesis we preformed three experiments. In the first experiment, we determined if blocking $GABA_A$ receptors in the mPFC of postpartum females would prevent the normal reduction of anxiety. In the second experiment, we examined the extent to which activating $GABA_A$ receptors in the mPFC could restore postpartum anxiolysis. Last, we investigated if dams who exhibit elevated anxiety-like behavior as a consequence of pup separation would also display a reduction in GABAergic neuron activation in the mPFC as compared to mothers displaying low anxiety.

Methods

Animals

Virgin female (200–250 g) and male (300–350 g) Sprague Dawley rats from Taconic (Germantown, NY) arrived at our facility and were individually housed for 1 week of acclimation. All rats were housed in a temperature and humidity controlled room and maintained on a 12/12 light/dark cycle (lights on at 0600 hr) with access to food and water ad libitum. All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee.

For breeding, one male was placed with one virgin female in their home cage. Pregnancies were verified through daily vaginal swabs and microscopic identification of sperm. Upon positive determination of pregnancy, designated as gestation day (GD) 0, female rats were individually housed. The day of birth was designated as postpartum day (PD) 0, and on PD1, litters were culled to 8 pups (4 males, 4 females).

In virgin females, stages of estrous were monitored through daily vaginal swabs which were taken at least 2 hr prior to testing. Samples of cells were obtained with a sterile cotton swab saturated in 0.9% saline and applied to a glass slide. After drying, slides were stained with 1% aqueous Toluidine Blue and cell types characterized under 10X magnification (Everett, 1989). Only those virgin females that had normal 4-5 day estrous cycles were used. All animals were weighed daily.

Surgical procedures

Virgin or pregnant (GD16-18) rats were anesthetized with a 2-4% isoflurane gas/air mixture and aligned on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). This timepoint for surgery is consistent with prior studies assessing behavioral changes during the postpartum period following drug administration via cannulation (Neumann et al., 1999; Lubin et al., 2003; Figueira et al., 2008). Body temperature was maintained throughout the surgery with a warming pad. Bilateral cannula guides (pedestal mounted 22-gauge stainless steel tubes with 1.5 mm separation and cut 3.5 mm below the pedestal; Plastics One, Roanoke, VA) were secured in a stereotaxic holder and lowered into the prelimbic region (PL) of the mPFC (AP: + 3.2 mm, ML: ± 0.75 mm, DV: -3.2mm; Paxinos and Watson, 1998). The PL mPFC was targeted because it has been consistently linked to maternal anxiety (Febo et al., 2010; Febo, 2012; Nephew et al. 2009; Pereira and Morrell, 2011). The cannula were secured by stainless steel screws and dental cement. A bilateral stainless steel obturator (0.35 mm diameter; Plastics One) extending 0.2 mm beyond the tip of the guide cannula was placed into the guide cannula after surgeries. The scalp was closed around the protruding portion of the cannula with sutures. On PD2, dams underwent a 30 min maternal behavior test to ensure that cannulation surgery did not interfere with maternal behavior. Only females who retrieved all of their pups to a common nest site, nursed and groomed the pups within the 30 min test, and whose pups gained weight were retained in the study.

Central infusions

On day 3 post-surgery all females were habituated to the handling and infusion procedures. Postpartum rats were again habituated to the handling and infusion process on PD6 while virgin females were habituated a second time at least one week after surgery on a day of estrus, with the

assumption being that they would be in diestrus the next day, which was confirmed after behavioral testing via vaginal swabbing. During habituation, rats were removed from their home cage and handled for approximately 3 min while being lightly restrained in a terrycloth towel. The obturators were then removed and a 28-gauge bilateral injection cannula extending 0.2 mm beyond the tip of the guide cannula into the PL cortex was inserted into the guide. The injection cannula were left in place for 3 min then removed and the obturator replaced. On testing days, all females were brought into the infusion room in their home cage. Following a 20 min habituation period, rats underwent the same procedure as described above except that an injection cannula attached to two 1 µl Hamilton Syringes via PE-10 tubing was inserted into the guide cannula. Bilateral infusions were made using a Harvard Apparatus Pico Plus Elite infusion pump (Holliston, MA) which delivered a 0.5 µl volume into each hemisphere over 3 min. The injector was left in place for an additional 1 min before withdrawal. Rats were then returned to their home cage which was then placed in an adjacent room for testing. 10 min after infusion, rats were tested on the elevated plus maze (EPM).

Experimental design

Experiment 1. To determine whether blockade of $GABA_A$ receptors in the PL mPFC can increase postpartum anxiety, virgin and postpartum female rats received bilateral infusions of 0.5 µl saline (n = 5 postpartum; n = 7 virgin) or 2.5 ng bicuculline methiodide (BIC; n = 7 postpartum; n = 6 virgin) dissolved in 0.5 µl of saline into the PL mPFC. These doses were based on previously published work (Miller et al., 2010). Anxiety-like behavior was assessed on PD7 in postpartum females and during diestrus in virgin females in order to control for fluctuations in anxiety across the estrous cycle (Mora et al., 1996; Marcondes et al., 2001; Walf and Frye, 2007). Studies which

have examined factors regulating anxiety-like behavior in virgin females commonly test during diestrus since this is the stage in which anxiety-like behavior is relatively stable (Marcondes et al., 2001; Figueira et al., 2008).

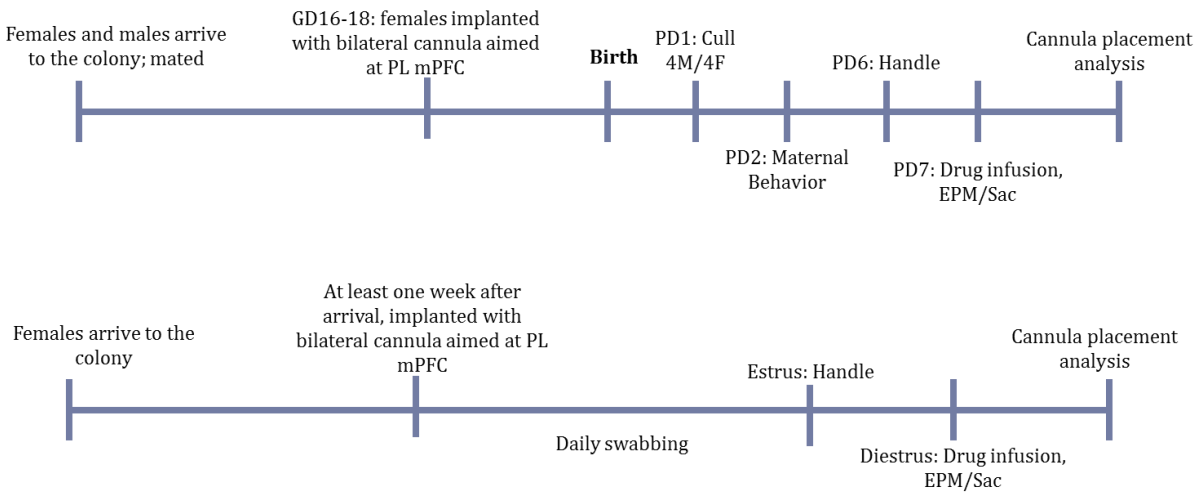

Figure 1: Experiment 1 timeline for postpartum (top) and virgin (bottom) females.

Experiment 2. In experiment 2, we examined whether activation of $GABA_A$ receptors in the PL mPFC of dams that have been separated from their pups can restore the reduced levels of anxiety-like behavior that are typically observed during this time. A two-factor design was used with litter presence before testing as one factor (no separation or 4 hr separation) and subsequent drug infusion type as the other factor (saline or muscimol), resulting in a total of 4 groups: no separation/saline, 4 hr separation/saline, no separation/muscimol, 4 hr separation/muscimol. On PD7, approximately half of the postpartum females had their pups removed and placed in an incubator set at 34 °C (nest temperature) 4 hr before testing. This separation time has been previously found to increase postpartum female rats' anxiety-related behavior in an EPM (Lonstein, 2005; Figueira et al., 2008; Smith and Lonstein, 2008; Miller et al., 2011). The other half of dams had the experimenter's hand placed in their home cage, the pups picked up, and

immediately replaced to control for perturbation caused by longer-term litter removal in females from the other condition. Otherwise, postpartum females from the no separation group were left in their home cages and allowed continual contact with their pups until the time of infusion. Dam and litter manipulation occurred between 0600-0700 hr on PD7. 4 hr after pup separation or 4 hr after the morning litter manipulation, postpartum females from both groups (with pups and pups removed) received either 0.5 μl saline (n = 6 no separation; n = 5 4hr separation) or 0.5 μg muscimol (n = 6 no separation; n = 6 4hr separation), a $GABA_A$ receptor agonist, dissolved in 0.5 μl of saline into each hemisphere of the PL mPFC. These doses are based on previously published work (Shah et al., 2004; Chan et al., 2011; Maeng and Shors, 2013; Solati et al., 2013).

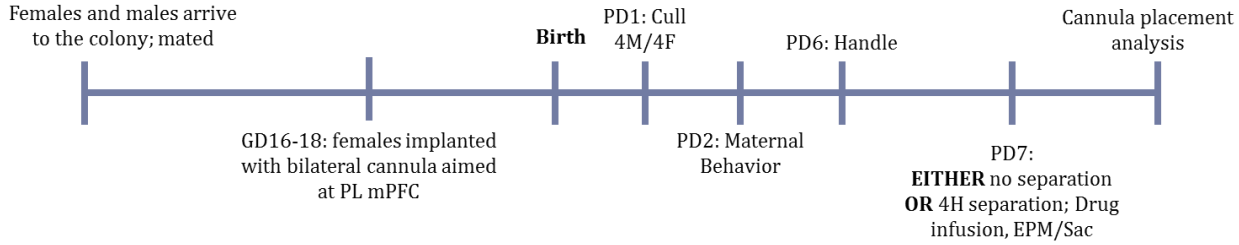

Figure 2: Experiment 2 timeline.

Experiment 3. In experiment 3, we examined the effects of pup separation on activation of GABAergic neurons in the PL mPFC following exposure to the EPM. On PD7, one group of postpartum females (n = 11) had their pups removed and placed in an incubator set at 34 °C (nest temperature) 4 hr before testing. The other group of dams had the experimenter's hand placed in their home cage, the pups picked up, and immediately replaced to control for perturbation caused by longer-term litter removal in females from the other condition (n = 12). Otherwise, dams from the no separation group were left in their home cages and allowed continual contact with their pups until the time of infusion. Dam and litter manipulation occurred between 0600-0700 hr on PD7. 4

hr after pup separation or after the morning litter manipulation, postpartum females from both groups were habituated to the testing room for 10 min prior to EPM testing. After testing, dams were returned to their home cage (which contained pups or not) which was carried back to the colony. 60 min after behavioral testing all females were euthanized and brains removed for immunohistochemical analysis as described below.

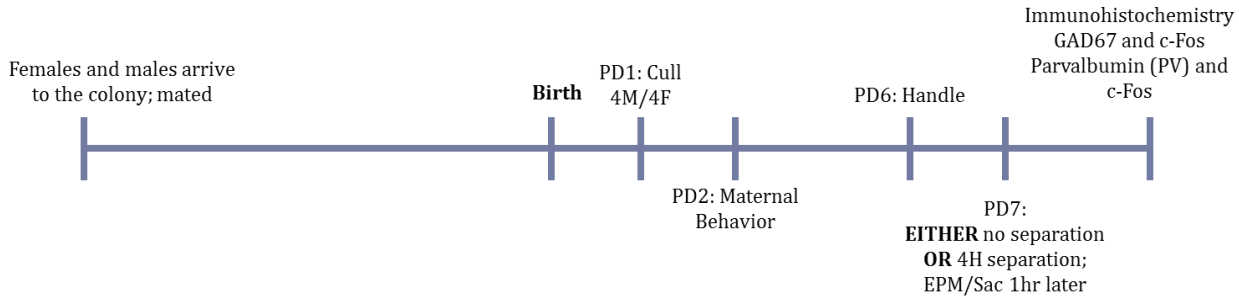

Figure 3: Experiment 3 timeline.

Anxiety-like behavior

The EPM was used to test for anxiety-like behavior (Prut and Belzung, 2003; Lapiz-Bluhm et al., 2008; Rotzinger et al., 2010). The EPM consisted of a cross-shaped platform (height: 50 cm) with four arms (width: 10 cm, length: 50 cm), two of which were enclosed by walls 50 cm in height. Rats were placed in the center of the platform (10 x 10 cm), facing a junction between an open and closed arm and allowed to explore for 5 minutes. The number of entries into the open arms and the percentage of time spent in the open arms (time in open arms/time in open and closed arms x 100) were used as measures of anxiety-like behavior (Pellow et al., 1985; Cruz et al., 1994; Lapiz-Bluhm et al., 2008). An increase in the percentage of time spent in the open arms and a greater number of open arm entries are indicative of reduced anxiety. Closed arm entries were used as a measure of locomotion independent of anxiety (Pellow et al., 1985; Cruz et al., 1994; Lapiz-Bluhm et al., 2008). The EPM measures of anxiety and locomotion analyzed are consistent with

numerous other studies investigating anxiety like-behavior including those studying motherhood (Pellow and File, 1986; Silva et al., 1997; Walf and Frye, 2007; Yang et al., 2015). In all cases, behavioral testing was done between 0900-1200 hr.

Histology

For experiments 1 and 2, rats were overdosed with Euthasol and transcardially perfused with 4% paraformaldehyde 24-48 hr after the completion of behavioral testing. Brains were removed, postfixed for 24 hr and then sectioned on a Vibratome. 40 µm thick coronal sections were collected throughout the area of the cannula implant and stained with 0.2% cresyl violet for verification of correct placement (Fig. 1). Those animals with cannula placements outside of the PL region of the mPFC (2 virgin females from experiment 1 and 1 postpartum female from experiment 2) were excluded from the study. Examination under high magnification (100X) revealed limited to no damage at the tip of the cannula in any of the animals.

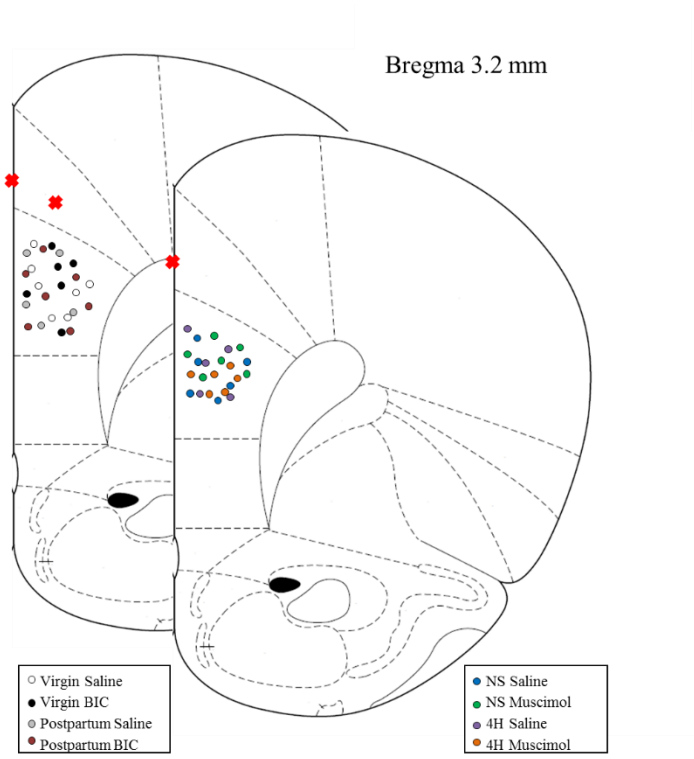

Figure 4: Schematic representation of mPFC cannula placements for Experiment 1 and 2. Cannula tip placements were in the prelimbic region (PL) of the mPFC (AP: +3.2 mm, ML: ±0.5 mm, DV: -3.2 mm). Each dot indicates an individual subject. Infusions were bilateral but are represented unilaterally. Cannula placements for virgin and postpartum females receiving an infusion of 2.5 ng BIC, or saline (left). Cannula placements for postpartum females receiving an infusion of 0.5 ug muscimol, or saline and either having no separation from their pups or 4 hr of separation (right). Animals with missed cannula placements in Cg1 or the ventricle (indicated by a red X) were excluded from analysis. Adapted from Paxinos and Watson, 1998.

For experiment 3, subjects were overdosed with Euthasol and transcardially perfused with 4% paraformaldehyde 1 hr after EPM testing. This time point was based on the maximal Fos protein expression in the rat brain following exposure to the EPM (Duncan et al., 1996). Brains were postfixed overnight at 4 °C. On the following day, brains were transferred to 30% sucrose in 0.1M PBS until sectioning. 50 µm sections extending through the mPFC (AP: 4.2 – 2.0mm) were obtained with a cryostat and stored in a sucrose-based cryoprotectant at -20ºC until immunohistochemical processing on free-floating sections. Four sections (1:6) were processed for double-labeling for GAD67 and c-Fos as well as PV and c-Fos. Briefly, sections were washed in

0.1M PBS (3x5min) and then incubated with 0.1% Tween in PBS for 10 min. Next, sections were blocked in 10% normal goat serum (NGS) and 0.3% Triton X in PBS for 60 min followed by incubation in rabbit anti-Fos primary antibody (1:300; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 1% Triton X and 3% NGS for 72 hr at 4°C. After a PBS rinse, sections were incubated for 2.5 hr at room temperature in goat anti-rabbit secondary antibody with Alexa 488 (1:500; Vector Laboratories, Burlingame, CA) and rinsed in PBS.

For co-labeling with GAD67, sections were then incubated in mouse anti-GAD67 primary antibody (1:2000; Millipore, Billerica, MA) in PBS with 0.5% tween overnight at 4°C. After a PBS rinse, sections were incubated for 1 hr at room temperature in DyLight 549 horse anti-mouse secondary antibody (1:500; Vector Laboratories, Burlingame, CA). For co-labeling with PV, sections were blocked in 0.3% Triton X and 10% normal horse serum (NHS) for 60 min at room temperature before being incubated overnight at 4°C in mouse anti-PV primary antibody (1:100; Millipore, Billerica, MA) diluted in PBS with 1% Triton X and 3% NHS. After a PBS rinse, sections were incubated for 2.5 hr at room temperature in DyLight 549 horse anti-mouse secondary antibody (1:500; Vector Laboratories, Burlingame, CA). After a final rinse in PBS, all sections were mounted on SupraFrost Plus microscope slides, coverslipped with DABCO, and kept in the dark at 4°C until imaging.

Quantification

For confocal analysis, a Nikon 90i microscope was used to obtain 10x (PV+/c-Fos+) and 20x (GAD67+/c-Fos+) image stacks of the mPFC from PD7 mother rats (~100 steps in 0.3 μm intervals along the z-plane). Image stacks were then projected using NIS Elements software and total number of c-Fos+, GAD67+, and PV+ cells as well as GAD67+/c-Fos+ and PV+/c-Fos+

double-labeled cells in the PL mPFC were counted by an researcher blind to experimental conditions. Identification of this region was conducted with reference to illustrations from a standard stereotaxic rat brain atlas (Paxinos and Watson, 1998) and landmarks such as the location of the corpus callosum. For the GAD67 analysis, 4 unilateral images of the PL mPFC were taken at 20x and 4 counts were performed unilaterally in a 250000 μm^2 ROI. For the PV analysis, 6-8 unilateral images of the PL mPFC were taken at 10x and 6-8 cell counts were performed from these sections in a 160000 μm^2 ROI. Percentages of GAD67+ and PV+ cells expressing c-Fos were calculated by dividing the number of these cells expressing c-Fos by the total number of single-labeled cells for the respective neurochemical marker. For each ROI, counts and percentages were divided by the area of the ROI then averaged for each animal and the group mean determined from these values. Data are expressed as the number of immunoreactive cells per 1 mm^2. Two brains from the 4h separation group were excluded due to an insufficient number of sections for analysis.

Statistical analysis

All statistical analyses were performed using Graphpad Prism software version 5.01 (La Jolla, CA). Anxiety-like behavior for Experiments 1 and 2 was analyzed using a two-way Analysis of Variance (ANOVA) with reproductive state (postpartum or virgin) or pup interaction (no separation or 4h separation) and infusion type (saline, BIC, or muscimol) as factors. Anxiety-like behavior and immunohistochemical data from Experiment 3 was analyzed using a two-tailed Student's t-test. Statistical significance for main effects and interactions were indicated by p values < 0.05 and when significance was found were followed by Tukey's HSD post hoc comparison test.

Results

Experiment 1: Attenuated anxiety during the postpartum period is prevented by blocking $GABA_AR$ in the mPFC

For the percentage of time spent in the open arms of the EPM (Figure 2a), there was a main effect of reproductive state such that postpartum females spent more time in the open arms than virgins ($F_{1, 21} = 6.31$, $p < 0.05$) indicating lower anxiety in postpartum females. There was also a significant main effect of infusion type ($F_{1, 21} = 6.76$, $p < 0.05$) and a significant reproductive state by infusion type interaction ($F_{1, 21} = 7.23$, $p < 0.05$). It was determined via post hoc analysis that the group receiving BIC spent less time in the open arms compared to the saline infused postpartum group (p's < 0.05). Postpartum females given BIC did not differ from virgins in the percentage of time spent in the open arms (p's > 0.05). No differences were detected between the virgin groups (p's > 0.05).

For the number of entries made into the open arms of the EPM (Fig. 2b), there was also a significant main effect of reproductive state such that postpartum females made more open arm entries than virgins again indicating reduced anxiety in postpartum females ($F_{1, 21} = 14.90$, $p < 0.05$). There was also a significant main effect of infusion type ($F_{1, 21} = 12.90$, $p < 0.05$) as well as a significant reproductive state by infusion type interaction ($F_{1, 21} = 10.75$, $p < 0.05$). It was determined via post hoc analysis that in postpartum rats, the group receiving BIC had fewer open arm entries compared to the saline infused postpartum group (p's < 0.05). Postpartum females given BIC did not differ from virgins in the percentage of time spent in the open arms (p's > 0.05). No differences were detected between the virgin groups (p's > 0.05).

There was no main effect of reproductive state ($F_{1, 21} = 2.02$, $p > 0.05$) or infusion type ($F_{1, 21} = 0.12$, $p > 0.05$) and no significant reproductive state by infusion type interaction ($F_{1, 21} = 0.05$,

p > 0.05) for the number of closed arm entries in the EPM (Fig. 2c), indicating that locomotor activity was not altered by reproductive state or infusion type.

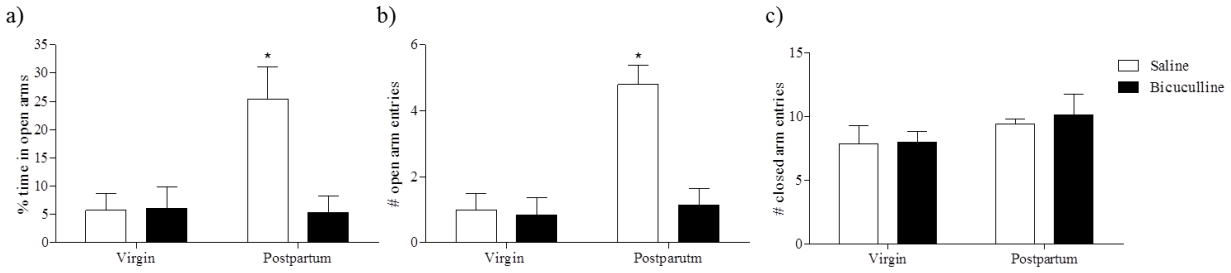

Figure 5: Blocking $GABA_AR$ in the mPFC enhances postpartum anxiety, but has no effect on anxiety in virgin females. Postpartum females infused with saline in the mPFC spent a greater percentage of time in the open arms (a) and made more open arm entries (b) as compared to virgins and BIC infused dams. In contrast, postpartum females receiving BIC displayed a decrease in the percentage of time spent in the open arms (a) and made fewer open arm entries (b) as compared to saline infusion dams and did not differ from virgin groups. Locomotor activity, as measured by the number of closed arm entries (c), was not altered. Neither of the virgin groups differed significantly from one another on any measure (a,b,c). Bars represent mean + SEM; * $p < 0.05$ vs all other groups.

<u>Experiment 2</u>: Attenuated anxiety during the postpartum period is prevented by mother-pup separation and restored by agonizing $GABA_AR$ in the mPFC

While there was no main effect of pup separation ($F_{1, 19} = 2.30$, $p > 0.05$), there was a main effect of infusion type ($F_{1, 19} = 8.12$, $p < 0.05$) and a significant interaction between pup separation and infusion type ($F_{1, 19} = 7.48$, $p < 0.05$) for the percentage of time spent in the open arms of the EPM (Fig. 3a). Post hoc analysis revealed that in the 4 hr separation group, postpartum rats receiving saline spent less time in the open arms compared to the muscimol infused postpartum group ($p < 0.05$), indicating increased anxiety-like behavior. Postpartum females that were separated from their pups and then given muscimol did not differ from postpartum females that remained in contact with their pups in the percentage of time spent in the open arms (p's > 0.05). Dams in the no separation group did not significantly differ from each other ($p > 0.05$).

For the number of open arm entries in the EPM (Fig. 3b), there was no main effect of pup separation ($F_{1, 19} = 1.61$, $p > 0.05$). However, there was a main effect of infusion type ($F_{1, 19} = 4.96$, $p < 0.05$) and a pup separation by infusion type interaction ($F_{1, 19} = 4.96$, $p < 0.05$). Post hoc analysis revealed that in the 4 hr separation group, postpartum rats receiving saline had fewer open arm entries compared to the muscimol infused postpartum group ($p < 0.05$). Postpartum females that were separated from their pups and then given muscimol did not differ from postpartum females that remained in contact with their pups in the number of open arm entries (p's > 0.05). Dams in the no separation group did not significantly differ from each other ($p > 0.05$).

For the number of closed arm entries in the EPM (Fig. 3c), there was no main effect of pup interaction ($F_{1, 19} = 0.31$, $p > 0.05$) or infusion type ($F_{1, 19} = 0.08$, $p > 0.05$) and no significant pup interaction by infusion type interaction ($F_{1, 19} = 1.24$, $p > 0.05$) indicating that locomotor activity was unaltered by pup interaction or infusion type.

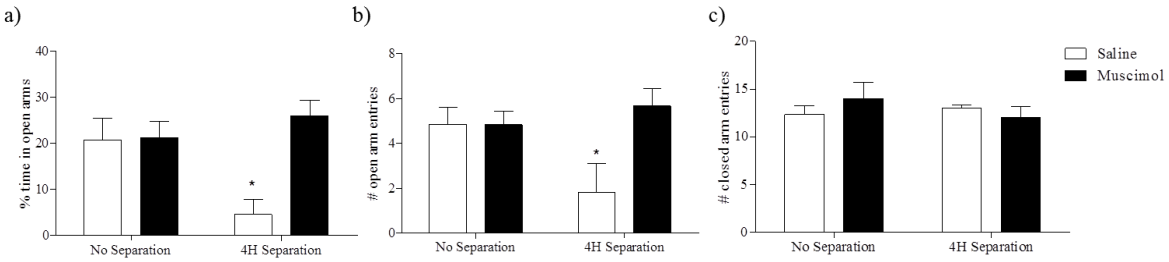

Figure 6: **Attenuated anxiety during the postpartum period is prevented by mother-pup separation and restored by agonizing $GABA_AR$ in the mPFC.** In the EPM**,** postpartum females that were separated from their pups for a period of 4 hr spent less time in the open arms (a) and made fewer open arm entries (b) as compared to all other groups indicating increased anxiety-like behavior. Muscimol into the PL mPFC reversed the anxiogenic effect of pup separation as evidenced by an increase in the percentage of time spent in the open arms (a) and more open arm entries (b). Locomotor activity, as measured by the number of closed arm entries, was not altered by pup separation or drug administration (c). Bars represent mean + SEM; *$p < 0.05$.

Experiment 3: Postpartum females that display low anxiety have a greater number of activated GABAergic neurons in the PL mPFC

Mother-pup separation increased anxiety-like behavior and altered GABAergic neuronal activation in the PL mPFC (Figure 4). In the EPM, mothers separated for a period of 4 hr from their pups spent less time in the open arms (Fig. 4a; $t(21) = 2.43$, $p < 0.05$) and had fewer open arm entries (Fig. 4b; $t(21) = 3.39$, $p < 0.05$) than mothers that were allowed continual contact with their pups. Locomotor activity, as measured by the number of closed arm entries, was not altered by pup separation (Fig. 4c; $t(21) = 1.56$, $p > 0.05$).

While mother-pup separation increased anxiety-like behavior, pup separation followed by EPM exposure did not alter the number of c-Fos+ (Fig. 4d; $t(19) = 1.55$, $p > 0.05$) or GAD67 (Fig. 4e; $t(19) = 0.49$, $p > 0.05$) cells within the PL mPFC. However, in mothers that displayed high anxiety levels due to pup separation, the number (Fig. 4f; $t(19) = 2.79$, $p > 0.05$) and percentage (Fig. 16g; $t(19) = 3.52$, $p < 0.05$) of GAD67+ neurons expressing c-Fos in the PL mPFC was reduced.

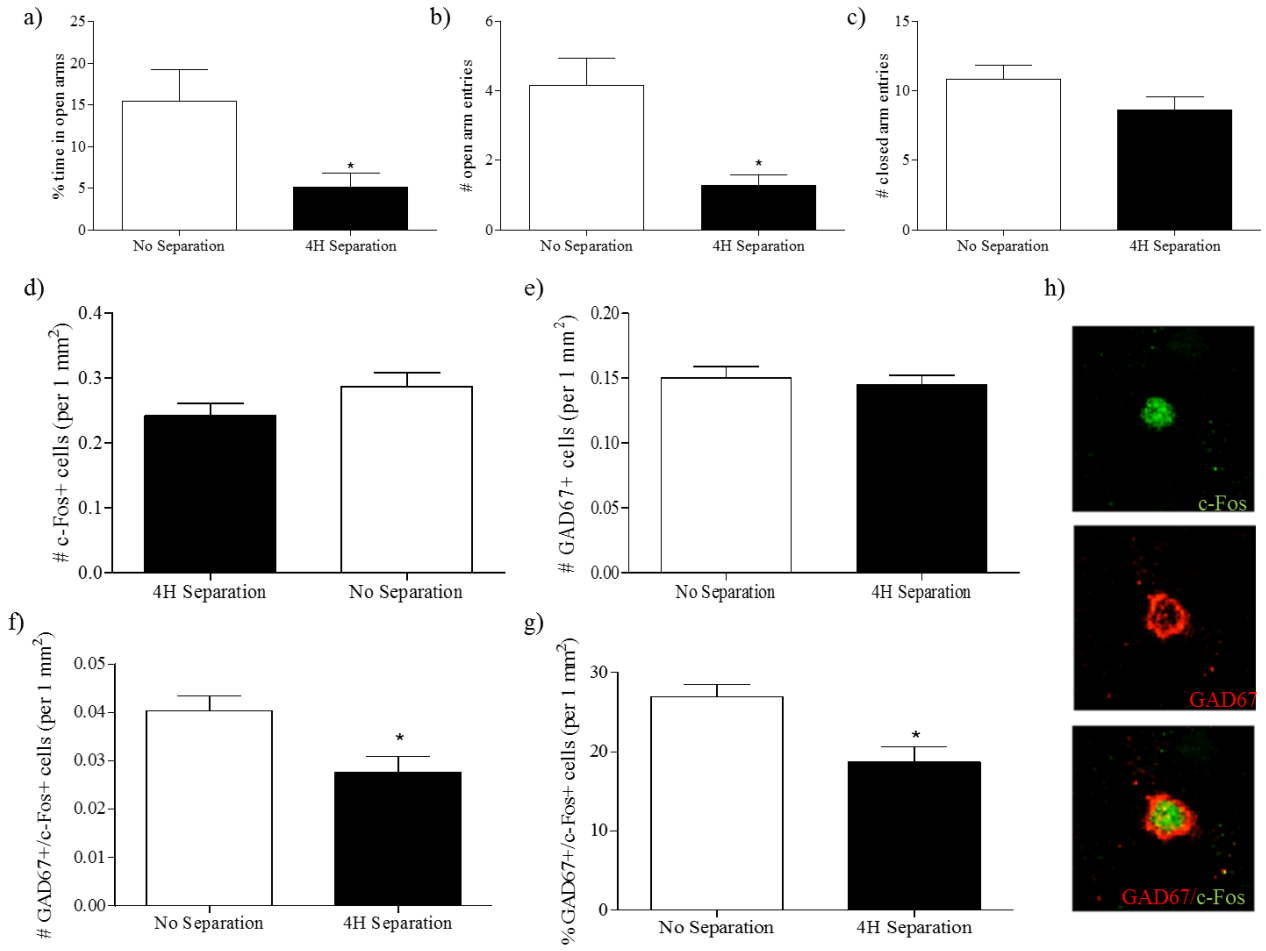

Figure 7: Postpartum females separated from their pups show increased anxiety in the EPM and increased GABA activation. Postpartum females separated from their pups spent less time in the open arms (a) and made fewer open arm entries (b) compared to mothers that were allowed continual contact with their pups. Locomotor activity, as measured by the number of closed arm entries (c), was not altered. Pup separation followed by EPM exposure did not alter the number of c-Fos+ (d) or GAD67+ (e) cells within the PL mPFC. However, in mothers with high levels of anxiety due to pup separation the number (f) and percentage (g) of GAD67+/c-Fos+ co-labeled cells in the PL mPFC was reduced. (h) Confocal images of a c-Fos+ (green, top), GAD67+ (red, middle), and GAD67+/c-Fos + cell (bottom) in the PL mPFC. Bars represent mean + SEM; *$P < 0.05$.

We further examined whether pup manipulation followed by EPM exposure specifically altered activation of a PV neurons, specific subtype of GABA interneuron, in the mPFC (Figure 5). Our results show that pup manipulation followed by EPM exposure did not alter the number of

c-Fos+ (Fig. 5a; t(19) = 0.50, p > 0.05), PV+ (Fig. 5b; t(19) = 0.19, p > 0.05), or double-labeled (c-Fos+ and PV+; Fig. 5c; t(19) = 0.25, p > 0.05) expressing cells within the PL mPFC. Additionally, pup separation did not affect the percentage (Fig. 5d; t(19) = 0.21, p > 0.05) of PV+ neurons expressing c-Fos in the PL mPFC.

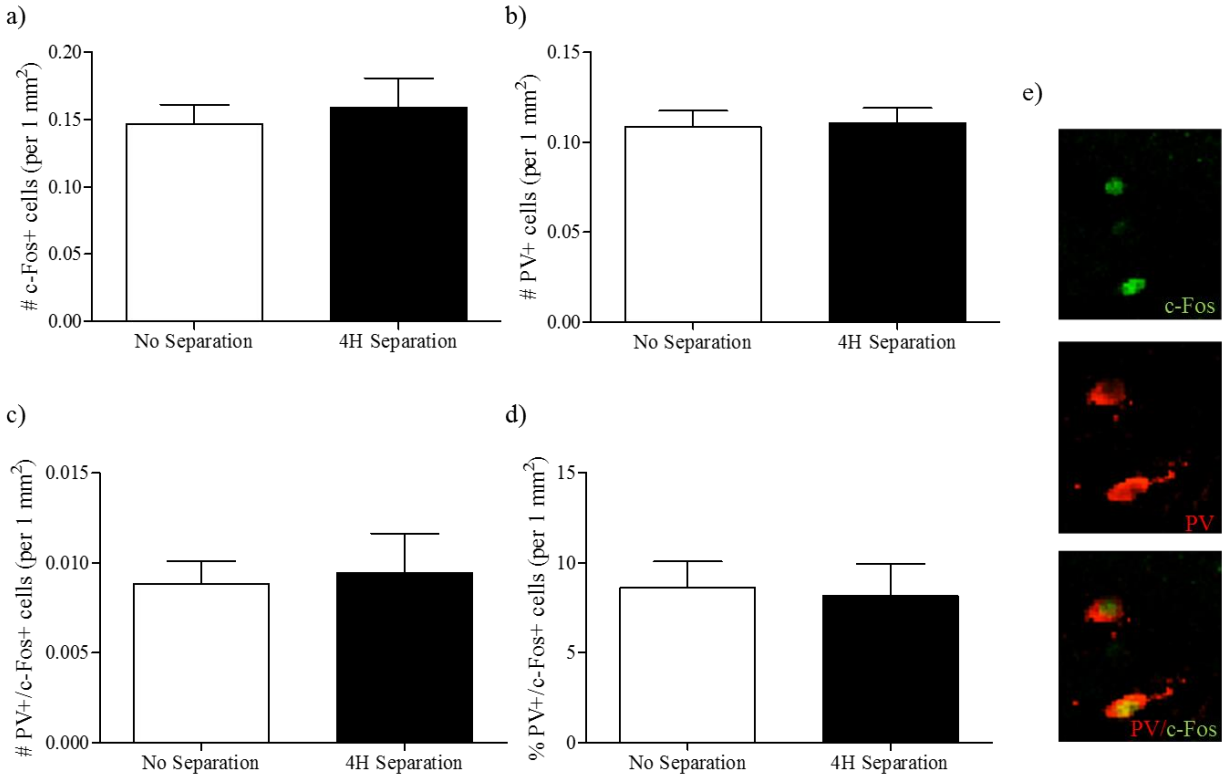

Figure 8: **Pup manipulation followed by EPM exposure did not alter activation of PV neurons in the PL mPFC.** Pup separation followed by EPM exposure did not alter the number of c-Fos (a) or PV (b) expressing cells within the PL mPFC. In addition, the number (c) and percentage (d) of PV-ir neurons expressing c-Fos in the PL mPFC was unaffected by pup separation. (e) Confocal images of a cell positive for c-Fos (green, top), PV (red, middle), and PV colabeled with c-Fos (bottom). Bars represent mean + SEM.

Discussion

The present work links the attenuation of anxiety-like behaviors in postpartum females to GABA signaling in the mPFC. Specifically, we show that blocking $GABA_AR$ in the PL mPFC prevents the normal reduction in postpartum anxiety without affecting anxiety in virgin females. Furthermore, we found that increased anxiety following mother-pup separation was accompanied by decreased activation of GABAergic neurons in the PL mPFC and that reduced maternal anxiety could be restored by activation of the $GABA_AR$ in the PL mPFC.

GABA has been related to changes in anxiety during the postpartum period (Lonstein et al., 2014). In laboratory rats, CSF concentrations of GABA are high in lactating rats that interact with pups, but are almost nondetectable in dams whose pups have been removed (Qureshi et al., 1987). Furthermore, administration of GABA agonists brings emotional responding in cycling female rats to a level similar to that found in postpartum females (Ferreira et al., 1989). Treating postpartum females with $GABA_A$ receptor antagonists peripherally or centrally (PAG) prevents the normal anxiolytic phenotype (Hansen et al., 1985; Miller et al., 2010). However, in regions including the ventromedial hypothalamus and amygdala, treatment with $GABA_AR$ antagonists does not seem to have any effect on postpartum anxiety-like behavior (Hansen and Ferreira, 1986).

Within the rat maternal mPFC, elevated GABAergic neurotransmission has been reported (Lonstein et al., 2014; Smolen et al., 1993). For example, microdialysis studies have demonstrated that lactating rats display elevated basal GABA release and turnover in the mPFC compared to virgin females (Arriaga-Avila et al., 2014). Additional data indicate that the mPFC of postpartum rats has higher expression of both GAD65 and the vesicular GABA transporter compared to diestrus virgins, suggesting greater potential for cortical GABA synthesis and release in mothers (Kornblatt and Grattan, 2001; Ahmed and Lonstein, 2012; Arriaga-Avila et al., 2014). While these

data collectively suggest that GABAergic neurotransmission in the PL mPFC may play a role in the modulation of anxiety during the postpartum period, this had not been explicitly tested.

Here, we show that infusion of BIC, a $GABA_A$ receptor antagonist, into the PL mPFC of postpartum females prevents the typical reduction in anxiety and brings emotional responding to a level that is similar to that of cycling virgin females. In contrast to postpartum females, BIC infused into the PL mPFC of diestrus virgins did not affect the percentage of time or number of open arm entries in the EPM, a finding that is in agreement with previous studies (Miller et al., 2010). The difference in sensitivity to BIC is likely not due to differences in the density of $GABA_AR$ in the mPFC as a study performed by Miller and Lonstein (2011) demonstrated that, within the mPFC, there are similar levels of GABA receptor binding in postpartum and virgin rats. However, it could be explained as a result of reproductive differences in GABA release and activation between virgin and postpartum females due to endogenous changes in ovarian hormones which result in increased inhibitory neurotransmission at the $GABA_AR$ in postpartum females (Perez et al., 1984; Concas et al., 1999; Toufexis et al., 2006; Frye, 2009). Thus, administration of BIC to virgin females would not increase the already high anxiety-like behavior because endogenous ligand levels are relatively low and antagonizing the $GABA_AR$ would have little effect. It may also reflect a floor effect such that the low duration of time diestrous virgin females spent in the open arms could not be decreased further.

Mother-pup interactions are known to be critical for reducing postpartum anxiety (Lonstein, 2005; Figueira et al., 2008; Smith and Lonstein, 2008; Miller et al., 2011). We replicate this by demonstrating that 4 hr mother-pup separation induces increased anxiety-like behavior in postpartum females, an effect which was accompanied by decreased activation of GABA neurons in the mPFC. Further we found that reduced anxiety was restored in pup-separated mothers by

administration of the $GABA_AR$ agonist, muscimol, to the PL mPFC. It is important to note that although we only examined the $GABA_AR$, two other subtypes of GABA receptors exist including $GABA_B$ and $GABA_C$ receptors. Most prior research has implicated activity at the $GABA_AR$ as a mediator of postpartum anxiolysis (Hansen et al., 1985; Hansen and Ferreira, 1986; Miller et al., 2010; Miller et al., 2011). Although the role of $GABA_B$ and $GABA_C$ receptors in postpartum anxiety has not yet been examined, some studies in non-maternal rats suggest an important role for the $GABA_B$ receptor in modulating anxiety-like behavior (Cryan and Kaupmann, 2005; Kumar et al., 2013). Thus, the potential exists that the $GABA_B$ receptor may also modulate postpartum anxiolysis.

Pup separation led to increased anxiety behavior accompanied with decreased activation of GABAergic neurons in the PL mPFC. However, a heterogenous population of GABA neurons exists within the mPFC that shape excitatory output including parvalbumin (PV), somatostatin (SOM) and vasoactive intestinal peptide (VIP) (McKlveen et al., 2015). Both PV and SOM interneurons inhibit targeted pyramidal cells, therefore decreasing excitatory output from the mPFC and possibly decreasing anxiety-like behavior (Gaykema et al., 2014). VIP interneurons are not likely to play a role in anxiety regulation as they are mainly disinhibitory, projecting radially to other interneurons (David et al., 2007; Pi et al., 2013; Gaykema et al., 2014). Interestingly, quantification of PV did not yield any significant differences between separated dams displaying high anxiety-like behavior vs undisturbed dams displaying low anxiety-like behavior. Although previous studies have implicated PV neurons in modulating anxiety-like behavior (Canetta et al., 2016), the possibility remains that pup-separation is affecting the SOM subtype in the mPFC as they too have been implicated in the regulation of anxiety (Albrecht et al., 2013; Han, 2013; vanova et al., 2014; Nakajima et al., 2014; Li et al., 2016).

In addition to GABA in the mPFC, there are various other neurochemicals acting on multiple brain sites which contribute to reduced maternal anxiety (Lonstein, 2007). These include the neuropeptide oxytocin (OT) acting within brain regions including the PAG, paraventricular nucleus, amygdala and mPFC (Blume et al. 2008; Figueira et al. 2008; Jurek et al. 2012; Sabihi et al. 2014). It is likely that GABA and OT have an interactive, if not synergistic relationship in regulating postpartum anxiety (Lonstein et al. 2014). For example, OT has been found to increase cortical GABA levels (Qi et al. 2012) and recent work has shown that within the cortex, OT receptors are located on GABAergic interneurons (Nakajima et al., 2014; Marlin et al., 2015). Further, GABA been linked to the anxiolytic action of exogenous OT within the amygdala (Huber et al., 2005; Knobloch et al., 2012) and PVN (Smith et al., 2016) of non-maternal rats. Taken together, it could be hypothesized that OT in the mPFC attenuates anxiety during the postpartum period by enhancing local GABA activity.

Despite the fact that postpartum anxiety affects 10-15% of the population in the Western world, it is not well understood. (Fairbrother et al., 2016; Dennis et al., 2017; Pawluski et al., 2017). The current results suggest that GABA signaling in the PL mPFC contributes to the postpartum suppression of anxiety-related behavior that is mediated by physical contact with offspring. Together, these findings provide insight into mechanisms that may become dysfunctional in high postpartum anxiety. With this knowledge, there is the possibility of developing better treatment strategies to ameliorate high maternal anxiety and the associated maternal care deficits in anxious mothers. In this way, this research has profound implications for mothers and their children.

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